ICCVAM Ocular Expert Panel Review

HET-CAM TEST
(HEN'S EGG TEST – CHORIOALLANTOIC MEMBRANE TEST)

TEST PROCEDURE OVERVIEW

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HET-CAM TEST - TEST PROCEDURE OVERVIEW

- History of the test
  - Basis were chicken-embryo models used by embryotoxicologists and virologists
  - HET-CAM test method first proposed by Luepke (1985) and Luepke and Kemper (1986)
  - 1988 start of a validation project (funded by the government of the Federal Republic of Germany)
    - Preliminary phase with test establishment and protocol development
    - Interlaboratory assessment with 35 substances in 12 laboratories
    - Database development with ca. 200 substances in 7 laboratories
  - 1992 publication of the HET-CAM test method as INVITTOX protocol No. 47 in the ERGATT/FRAME (UK) data bank of *In Vitro* Techniques in Toxicology
  - Additional evaluation/validation studies by e.g. CTFA and EC/HO

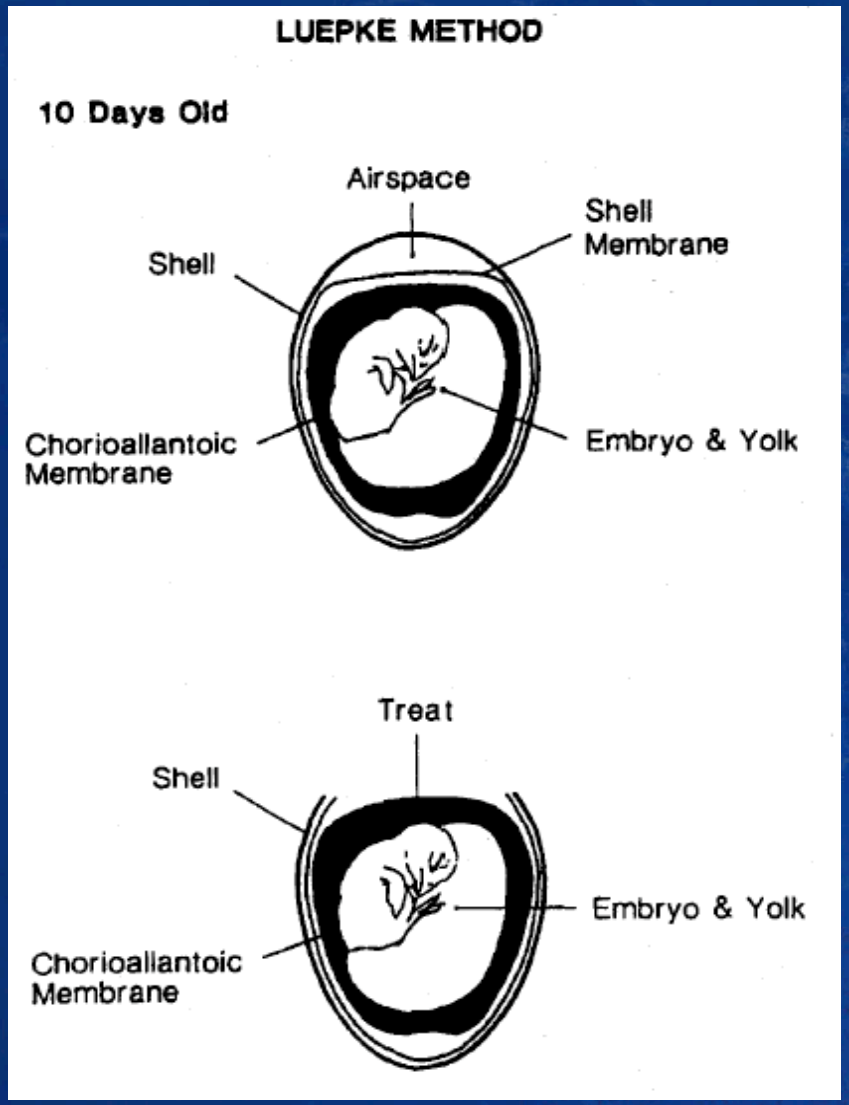
HET-CAM TEST - TEST PROCEDURE OVERVIEW

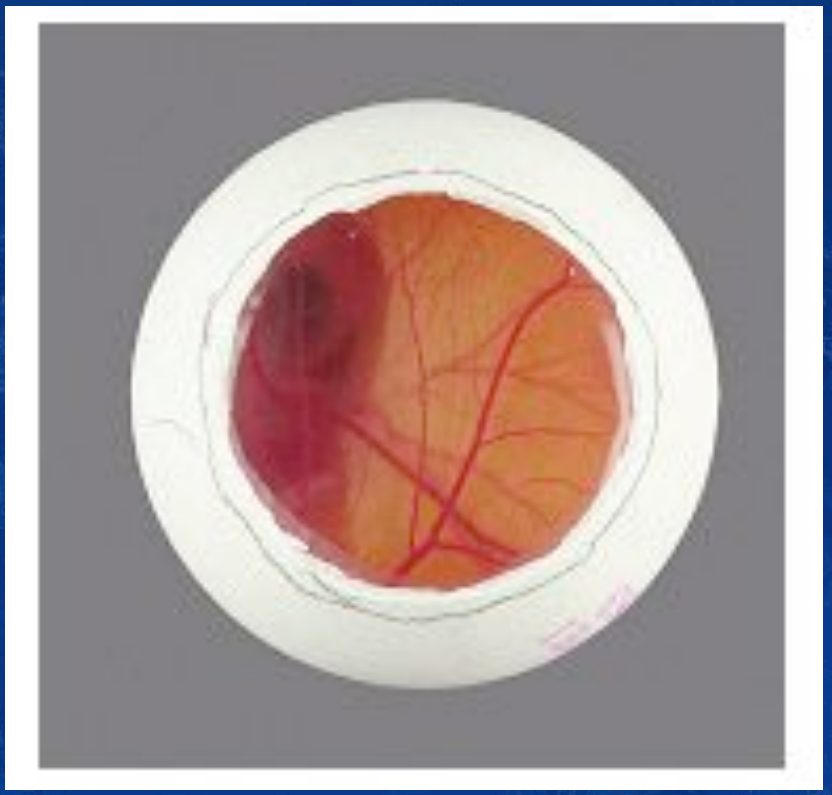
- Test principle
  - A defined dose of a test substance is applied to the chorioallantoic membrane (CAM) of fertilized and incubated hen's eggs
  - The CAM is evaluated for 300 sec for the development of defined endpoints
  - The time elapsed until the first appearance of the end-points is individually recorded **or**
  - Test substance is rinsed off the CAM at defined time-points (e.g. 30, 120, 300 sec) to evaluate and record changes if the physiocochemical properties of the test substance impair clear visualization
    -under discussion-

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- The chorioallantoic membrane
  - The vascularized respiratory membrane that surrounds the developing bird embryo; composed of
    - An ectodermal layer (epithelium – 2 to 3 cells thick)
    - A mesodermal layer (connective tissue, ground substance, blood vessels)
    - An endodermal layer (the allantoic sac composed of squamos cells)
  - The blood vessels that are present in the mesodermal layer of the CAM are branches from the embryo-allantoic arteries and veins; they form a capillary bed

HET-CAM TEST - TEST PROCEDURE OVERVIEW





HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Materials
  - White Leghorn hen's eggs
  - Fresh (not older than 7 days), fertile, clean eggs between 50 and 60 grams (eggs get candled and nonviable and defective eggs will be discarded)
  - Incubator with an automatic rotating device
  - Small saw or dentist rotary saw to remove the eggshell
  - Negative control substance: 0.9% (w/v) NaCl solution or olive oil or other vehicle and 0.9% (w/v) NaCl solution
  - Positive control substance: e.g. 10% NaOH, NaOH pellets, 1% sodium dodecyl sulfate (SDS)
  - Benchmark control
    -under discussion-

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Preparation of the test system
  - Incubation of the eggs for 9 days
    - Still-air incubator: 38.3 ± 0.2°C with a relative humidity of 58% ± 2%
    - Forced-air incubator: 37.8 ± 0.3°C with a relative humidity of 58% ± 2%
    - Eggs should be rotated at least 5 times daily for the first 8 days of incubation
  - Candling of the eggs after 8 days of incubation to ensure viability
  - Incubation for additional 24 hours without rotation
  - Prior to use removal of the eggshell along the air cell by means of a saw
  - Moistening of the white inner membrane with 0.9% (w/v) NaCl solution; Keeping eggs warm until use
  - Careful removal of the inner membrane immediately prior to use (not more than 20 min after removal of the eggshell

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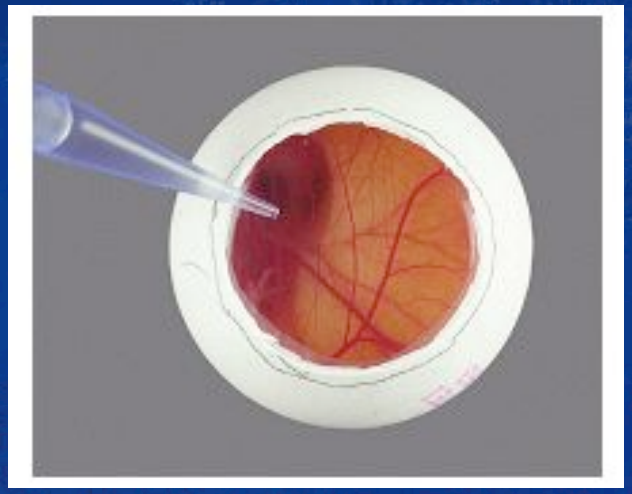

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Treatment with the test substance
  - Application of test substances directly to the CAM
    - Liquids: 0.3 mL
    - Solids, pastes or particulate substances: 0.3 mL, not more than 0.3 gram; solids to be grounded to a fine powder
  - Testing of the substances and formulations undiluted and in their original physical form; testing of lower concentrations if technical constraints (e.g. colored test substances) preclude clear CAM visualization or according to protocol
    - Covering of at least 50% of the CAM surface with test substance
  - Exposure of the CAM to the test substance for at least 300 sec

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Endpoints measured by visual inspection
  - (Hyperemia)
  - Hemorrhage
    Bleeding out of the blood vessels of the CAM with red blood dots around the vessels
  - Lysis
    Optical disappearance of small blood vessels in the CAM
    Cave: This is not a real lysis according to principles of general pathology. By stereomicroscopy it could be observed that in most cases this so called lysis is due to spasms of the small vessels which then do no longer contain blood. There are cases where after some time the vessels get refilled with blood. The “lysis” disappears.
  - Coagulation
    Thrombosis (intravascular dark spots), extravascular blood coagulation (dark spots), denaturation of albumen

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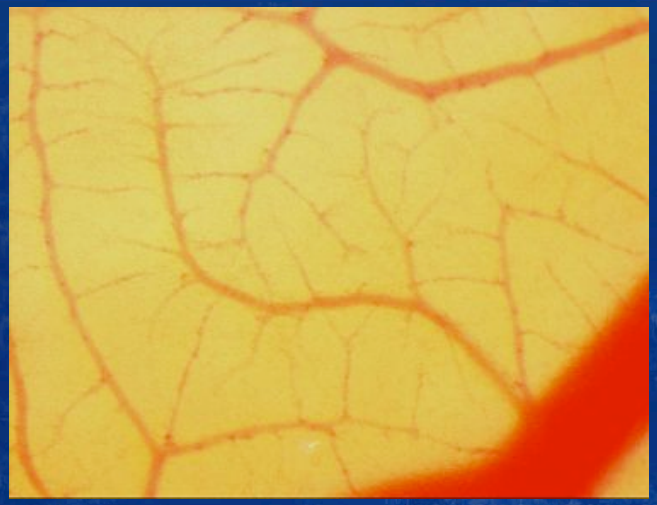
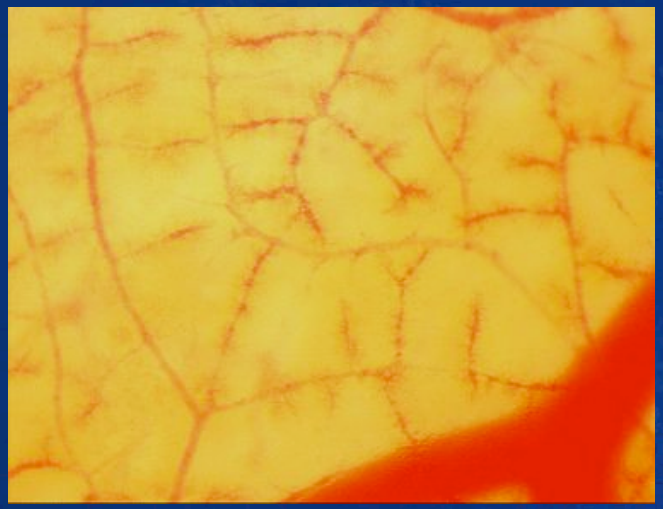
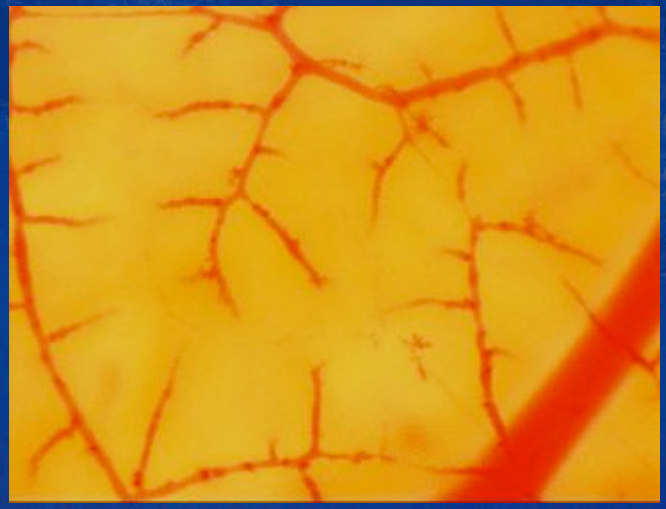


Prior to Test
Substance Treatment



Hemorrhage and Lysis



Coagulation (Thrombi)

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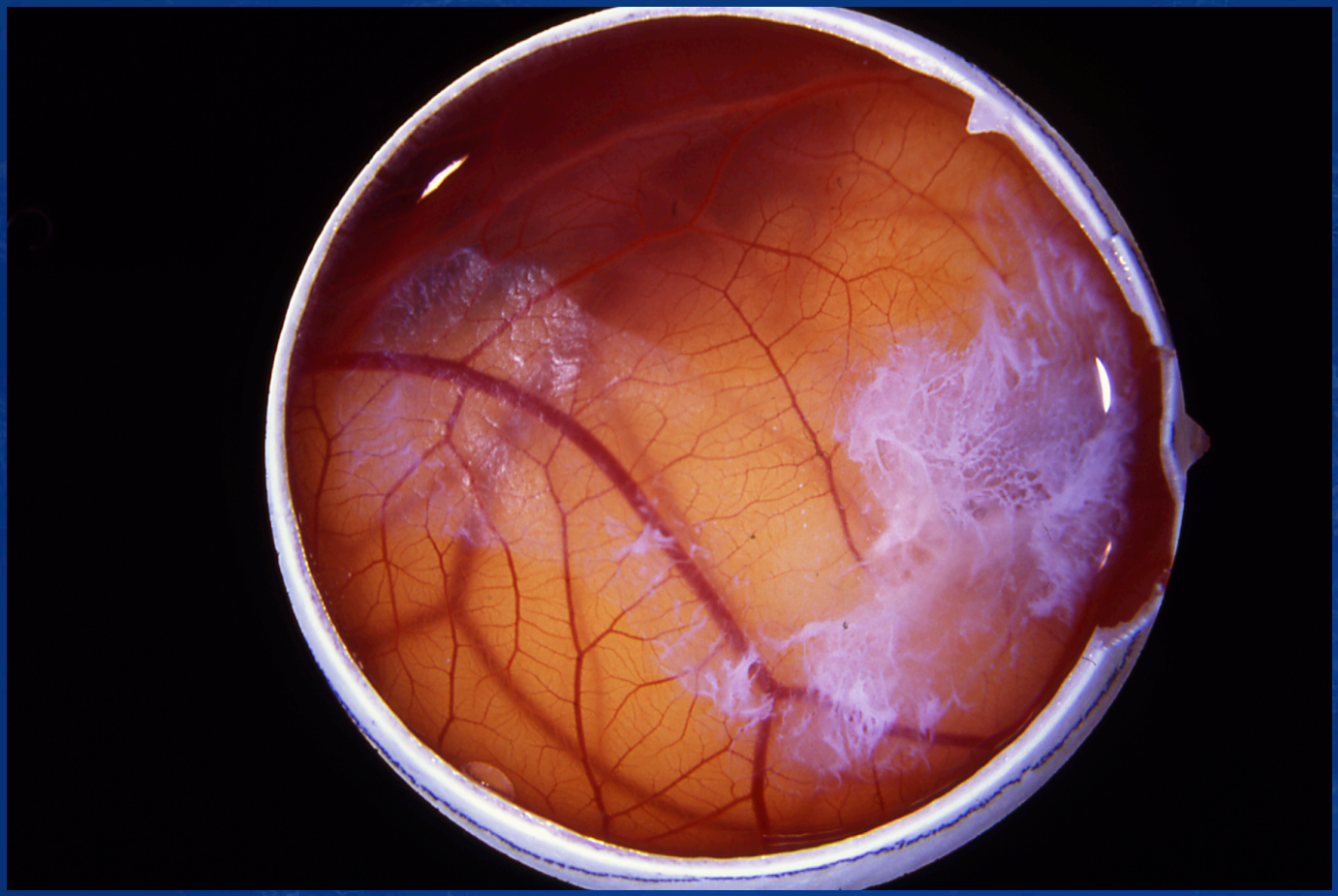

Coagulation of Albumen

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Data measured and recorded
  - Elapsed time between application of the test substance and the appearance of the endpoints, i.e. individually for each of the endpoints and for each egg (or 300 sec)
  - Severity of the main reaction after 300 sec
- Number of replicates
  - A minimum of 3 eggs per test substance (per concentration if dilutions have to be made), for each negative and positive and benchmark control, if used
- Repeat experiments
  - Not needed unless equivocal responses are observed in the 3 eggs tested

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Determination of the irritation threshold concentration (ITC) as the highest concentration at which slight reactions occur

- Calculation of the irritation score

$$\frac{(301 - \text{Hemorrhage time})}{300} \times 5 + \frac{(301 - \text{Lysis time})}{300} \times 7 + \frac{(301 - \text{Coagulation time})}{300} \times 9$$

Minimum score: 0

Maximum score: 21

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Decision criteria (examples for the decision “Severe Irritant / Corrosive”)
  - ITC < 1% **or**
  - 1.0% < ITC < 2.5% and IS (10%) > 16 **or**
  - 2.5% < ITC < 10.0% and IS (10%) < 16 **or**
  - IS (undiluted, not dissolved) ≥ 9.0 **or**
  - Mean detection time for appearing of coagulation (undiluted, not dissolved test substance) is < 60 sec

or…

HET-CAM TEST - TEST PROCEDURE OVERVIEW

- Study acceptance criteria
  - A test should be considered acceptable if the negative/ solvent and positive controls each give an IS value that falls within the appropriate classification; the same relates to benchmark controls (if used)
- GLP compliance
  - Studies should be conducted in compliance with current GLP guidelines (OECD, EPA, FDA)